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14. ABSTRACT
Inflammation is a defense mechanism to injury that contains infection and initiates tissue repair. A potential outcome of the inflammatory process is granulomatous inflammation characterized by a large collection of macrophages. In a previous study, we observed a low-grade inflammation around a pure collagen based scaffold on implantation into the rabbit patellar tendon. Additionally, the cross-sectional areas of the tendons treated with the implant were about 40% greater compared to the sham-operated controls. In the current study, we hypothesized that soft connective tissue such as tendon can be regenerated by creation of a granulomatous inflammation. To test this hypothesis, the collagen bioscaffold was implanted into the rat patellar tendon using a minimally invasive technique and the inflammation was blocked using liposomal clodronate. The control group animals were operated in a similar manner but did not receive the drug. The animals were euthanized 1 month post-implantation, tissues were harvested and tendon cross-sectional area and the area of the granulomatous inflammatory core was determined using quantitative histology. The results revealed that the presence of granulomatous inflammation was observed around the implant in the rat model similar to our previous results in the rabbit model. However, the tendon cross-sectional area was comparable between tendons treated with the implant and the sham-operated control. Furthermore, no difference in tendon area was observed between the experimental group (with clodronate) and control group (no clodronate). Together, these results refute the hypothesis of the current study possibly due to species related differences between rats and rabbits.

15. SUBJECT TERMS

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Introduction

Tendon injuries are highly common and pose a significant economic burden to the US (*1-3*). In the context of military combat operations, musculoskeletal injuries account for 60-70% of total injuries (*4*). Most of these injuries call for intense surgical procedure and extensive rehabilitation periods. Nevertheless, in a large number of cases, the healed tissue is poorly organized, weak and not fully functional due to significant loss in tissue mass. We recently reported that implantation of a pure collagen based bioscaffold within the tendon proper of a rabbit model results in a low-grade granulomatous inflammation around the implant and a 40% increase in tendon area (*5*). Based on this outcome, we hypothesized that soft connective tissues can be regenerated by the creation of a granulomatous inflammation. To test this hypothesis, the current study employed a rat model and the granulomatous inflammation post-implantation of the collagen bioscaffold was blocked by using liposomal clodronate to investigate whether the absence of granulomatous inflammation will negate the observed anabolism of the tissue. If a causation effect between the granulomatous inflammation and tendon area is demonstrated, controlled induction of granulomatous inflammation by relatively inert biomaterials can be exploited for the regeneration of atrophied tissue volume as a novel tissue engineering paradigm.

Body

Methods:

Synthesis of ELAC threads and ELAC Bioscaffolds:

ELAC threads and bioscaffolds were synthesized as previously described (*5-10*). Briefly, acid soluble monomeric collagen (Advanced Biomatrix, AZ) was dialyzed with distilled water for three days. The dialyzed collagen solution was loaded between two wires (electrodes) and an electric field of 12 volts was applied. In the presence of an electric field, the collagen monomers align along the isoelectric point and form highly oriented electrochemically aligned collagen (ELAC) thread. The ELAC thread was incubated with 10x PBS overnight at 37 °C. ELAC threads were recovered from the electrochemical cells and crosslinked with 0.625% genipin (in 70% ethanol) for 3 days at 37 °C. Three individual genipin crosslinked ELAC threads were manually braided to form the ELAC bioscaffold. The ELAC bioscaffold was sterilized in 70% ethanol, washed with DI water and incubated in culture medium (DMEM + 1% penic illin/streptomycin) overnight before surgically implanting it into the patellar tendon (PT) in a rat model.

Surgical Implantation and PT Collection:

For this project, three animal studies were performed; a pilot study to corroborate the clodronate dosage (n=2, euthanasia at 2 weeks; 2/2 clodronate), a pilot study to corroborate the patellar tendon enlargement following ELAC insertion (n=6, euthanasia at 4 weeks [3/6 clodronate, 3/6 no clodronate]), and a study to demonstrate that clodronate will reduce the tendon enlargement following ELAC insertion (n=10, euthanasia after 4 weeks; 5/10 clodronate and 5/10 no clodronate). In the final analysis, the results from all three studies were combined to obtain sufficient number of data points for statistical analysis.

Initially, male Sprague-Dawley rats were used (n=3; mean body weight 323 g.). However, they were too small for the procedure and for the remainder of the study, we used male Long-Evans rats (n=29, mean body weight 597 g). Rats were anesthetized with ketamine (40 mg/kg), xylazine (6 mg/kg) and maintained with oxygen and isoflurane administered via a mask. Rats were clipped and sterilely prepped for surgery. Surgical implantation of ELAC bioscaffold is shown in Fig. 1. Briefly, the right stifle was approached using a 2 cm medial parapatellar incision of skin and subcutaneous tissue, thus exposing the patella, the patellar tendon and tibial tuberosity. Following, an 18 gauge hypodermic needle was placed intra-tendinously from patella to tibial tuberosity. Then, the ELAC device was placed and anchored to the patella and tibial tuberosity using cyanoacrylate glue. The incision was also closed using cyanoacrylate glue. The procedure was repeated on the left stifle joint, however, without placement of the ELAC device (sham). Post-operative pain medication consisted of butorphenol (0.05 mg/kg) and buprenorphine (0.025 mg/kg).

Clodronate was injected intra-peritoneally under light anesthesia with isoflurane and oxygen. A loading dose (0.1 ml/10g IP) was administered 2 days before surgery and the maintenance dose (0.1 ml/20g IP) was administered from day 3 onwards every 5 days till euthanasia. The animals were euthanized at four weeks and the patella-PT-tibia units were isolated for analysis. The patella-PT-tibia units were fixed in 10% in neutral buffered formalin. Following decalcification the complex was embedded in paraffin. Five micrometer thin transverse sections were cut and stained with haematoxilin and eosin or Masson's trichrome stain.

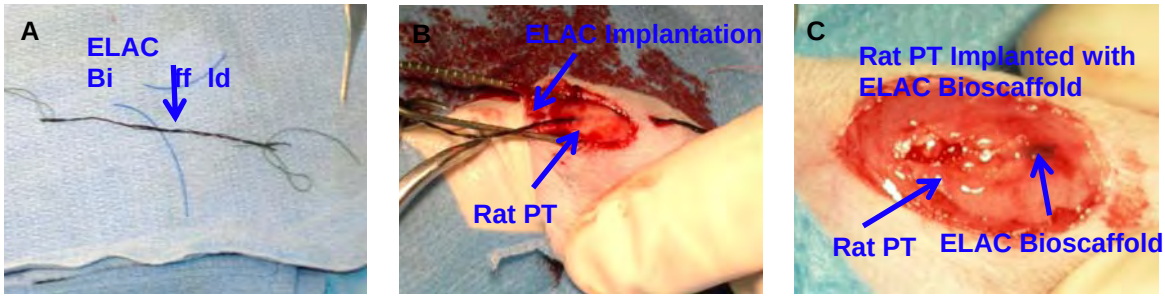


Figure 1: (A) An ELAC bioscaffold formed by braiding three individual ELAC threads together, (B) ELAC bioscaffold being surgically implanted into the rat patellar tendon (PT) and (C) Rat PT implanted with the ELAC bioscaffold.

Qualitative Histology Analysis:

The sections were evaluated qualitatively and semi-quantitatively by two observers (one boarded by the American College of Veterinary Pathologists). Sections were evaluated for presence of implant and inflammatory response. The inflammatory response was graded as none (0), minimal (1), mild (2), moderate (3), marked (4), and severe (5).

Quantitative Histology Analysis:

The Masson trichrome stained histological sections were used for the quantitative analysis of tendon cross-sectional area as described previously (*5*). Briefly, the tendon cross-sectional area and the granulomatous inflammatory area was determined by perimeter tracing of the Masson trichrome stained sections using Image J (U.S. National Institutes of Health, Bethesda, MD).

Statistical Analysis:

The tendon cross-sectional area data between the ELAC treated and sham-operated control PTs were analyzed using paired t-test. Mann-Whitney U test was used to analyze the clodonate vs. no clodronate effects on tendon area and granulomatous inflammatory area. Statistical significance was set at $p < 0.05$.

Results:

Histological analysis four weeks post-implantation showed the presence of a granulomatous inflammation in both groups with or without clodronate (Fig. 2). Qualitative evaluation of inflammation received an average score of 1 indicating mild level of inflammation in both groups (Fig. 3). Quantitative analysis revealed that the area of the inflammatory core was comparable with or without clodronate treatment indicating that intraperitoneal administration of clodronate was not effective enough to completely block inflammation within the tendon proper (Fig. 4B). Furthermore, the tendon cross-sectional area of the ELAC treated PTs was also comparable despite clodronate treatment. (Fig. 4A) Additionally, no difference in tendon cross-sectional area was observed between the ELAC treated and sham-operated PTs in both clodronate and no clodronate groups (Fig. 4A). These results suggest that the in vivo response to the ELAC bioscaffold in the rat model in terms of the type and level of inflammation was si milar to our previous results in a rabbit model (*5*) . However, unlike in the rabbit model, the increase in tendon area post ELAC treatment was not

observed in the rat model. This can be attributed to the species related differences in the defense mechanism between the rabbits and rats.

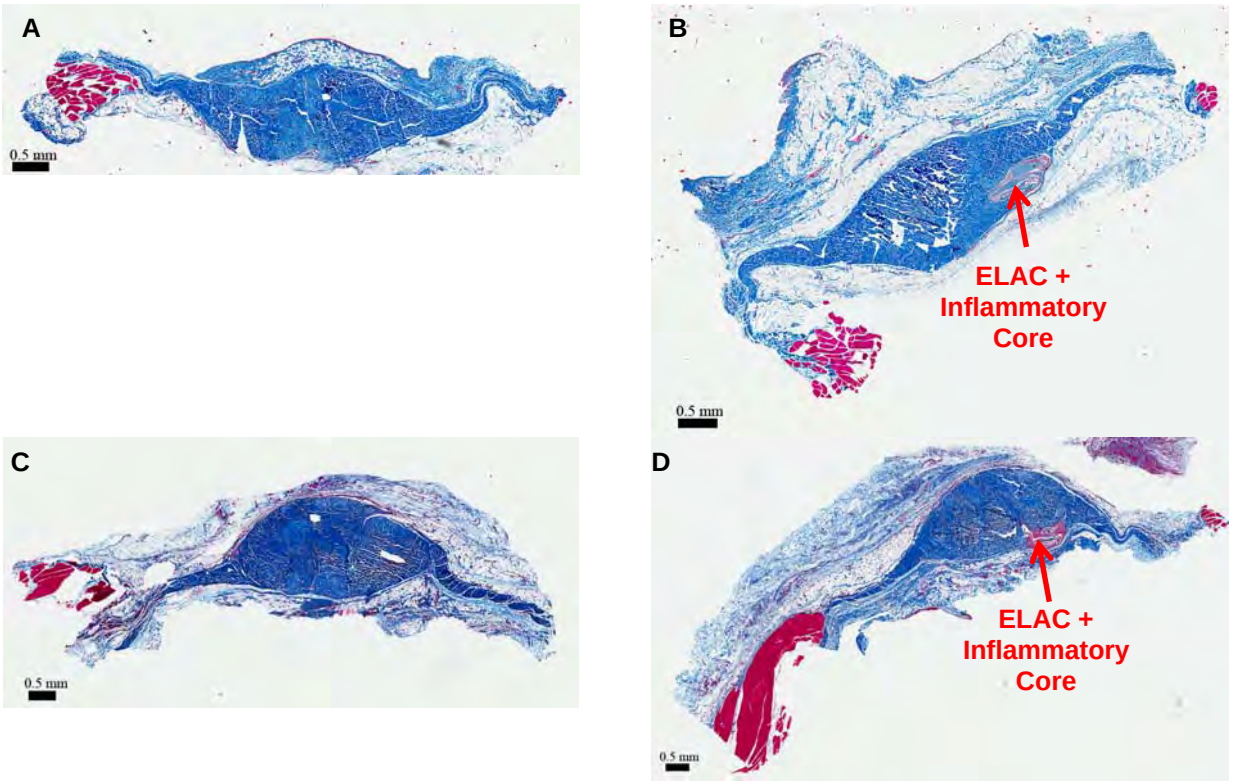


Figure 2: Masson trichrome stained histological images of the cross-section of rat PT. **(A)** control contralatera (no clodronate), **(B)** ELAC treated (no clodronate), **(C)** clodronate contralateral, **(D)** ELAC treated (with clodronate).

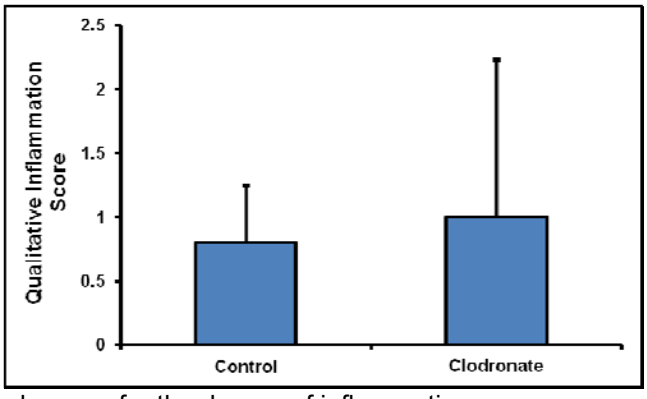
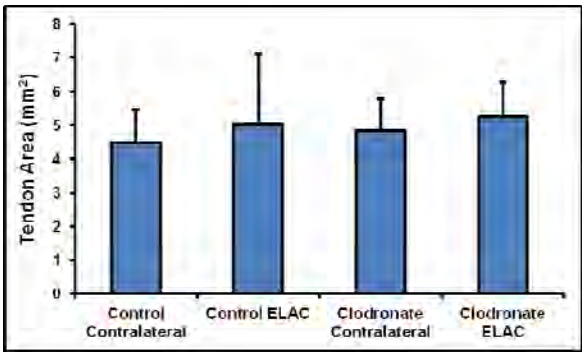


Figure 3: Graded histological scores for the degree of inflammation.



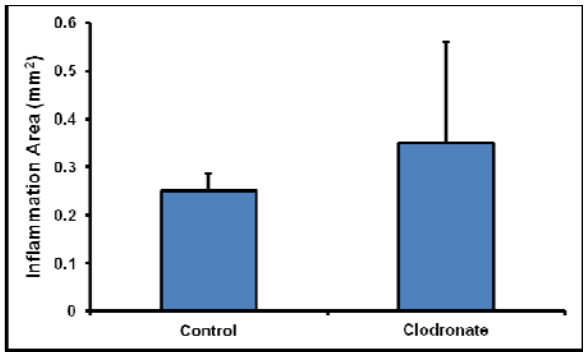


Figure 4: (A) Comparison of tendon crosssectional area between ELAC treated Control(no clodronate) and ELAC treated experimental (with clodronate) and their respective contralateral (sham-operated) PTs. **(B)** Comparison of inflammatory core area around ELAC bioscaffold between clodronate treated and control (no clodronate) rats.

Key Research Accomplishments:

1. The mild inflammatory reaction to ELAC in a rat model was similar to the one observed in our previous study using a rabbit model. This finding substantiates that ELAC is biocompatible.
2. Tendon regeneration typically takes months. The presence of the ELAC bioscaffold four weeks post-implantation suggests minimal degradation and confirms our previous results that ELAC degrades slowly. Therefore, it has the potential to be used as a biomaterial for tendon tissue engineering.
3. For rats and at one months, we have not observed the enlargement in the tendon area.

Reportable Outcomes:

Based on the negative outcome on hypothesis, we may have challenges in publishing these results. However, we are planning to sharing the results as an abstract in a conference platform during 2012.

Conclusion:

Unfortunately, our hypothesis that low-grade inflammation increases tendon area was refuted. There may be various reasons for this outcome. Ou r prior observations were from rabbits at 4 months. In the current study, due to limited budget (particularly due to cost of clodronate drug), we had to resort to a smaller animal (rat) and shorter duration (1 month). Therefore, for this species and at one month the hypothesis is refuted. However, we see trend for an increasing area with ELAC implantation by one month (Figure 4A), therefore, it may be possible that differences would emerge at a later point in time. While the hypothesis is not validated under stated conditions, we conclude that the hypothesis may be validated for longer durations, however, this would require greater resources.

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